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The arabidopsis gene Grassy, is required for auxin transport and patterning of leaf vein, shoot and root

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THE ARABIDOPSIS GENE GRASSY, IS REQUIRED FOR AUXIN TRANSPORT AND PATTERNING OF LEAF VEIN, SHOOT AND ROOT

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Submitted to the School of Graduate Studies of the University of Lethbridge in Partial Fulfilment of the Requirements for the Degree

MASTER OF SCIENCE

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I dedicate this thesis to my beloved wife Sushila and daughter Sadikshya
ABSTRACT

Auxin controls a range of growth related characteristics by a mechanism dependent upon polar auxin transport. We have identified a leaf vein patterning mutant that shows a simple first leaf vein pattern. The veins are often non-meeting and form somewhat parallel to one another. The leaves are narrow and pointed so that the overall leaf phenotype is reminiscent of grass leaves; hence the mutant name grassy (gsy). A range of shoot and root characteristics are also altered in gsy plants. Compared to wild type, gsy plants have shorter primary roots with reduced numbers of lateral roots and increased numbers of longer root hairs. Upon gravitropic stimulation, the root tip bends slightly away from the normal vector. As well, gsy plants produce an inflorescence with altered internode elongation and branching pattern. The intensity of the auxin responsive reporter gene DR5::GUS is unchanged in both roots and developing leaves of gsy, however, it shows subtle differences to the wild type DR5::GUS expression pattern. Finally, gsy leaf and root phenotypes are more sensitive to low doses of the auxin efflux inhibitor NPA and external auxin 2, 4-D. We suggest that this overall pattern is consistent with defects in auxin transport.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Genes and Protein Nomenclature

GSY  GRASSY wild type gene
gsy  GRASSY mutant allele
GSY  GRASSY protein product

Genes

ATHB8  ARABIDOPSIS THALIANA HOMEOBOX 8
ATMDR1  ARABIDOPSIS THALIANA MULTIDRUG RESISTANT1
ATPgp19  ARABIDOPSIS THALIANA P-GLYCOPROTEINS19
AUX1  AUXIN INSENSITIVE1
AXR1  AUXIN RESISTANT1
AXR2  AUXIN RESISTANT2
AXR6  AUXIN RESISTANT6
BDL  BODENLOS
CVP2  COTYLEDON VASCULAR PATTERN
DR5  DIRECT REPEAT
FKD1  FORKED1
GN/EMB30  GNOM
GUS  uidA
HVE  HEMIVENATA
LOP1  LOPPED1
MAX1  MORE AXILLARY BRANCHING
MP   MONOPTEROS
MYA2  MYASIN 2
PID   PINOID
PIN1  PIN FORMED
RMS   RAMOSUS
RTY   ROOTY
SFC   SCARFACE
VAN3  VASCULAR NETWORK3

Chemicals

2,4-D  2,4-Dichlorophenoxyacetic acid
EMS   Ethyl methane sulfonate
NAA   1-naphthyl-acetic acid
IAA   Indole-3-acetic acid
NaPO4 sodium phosphate
NPA   1-N-naphthylphthalamic acid
X-gluc 5-bromo-4-chloro-3-indolylglucuronide
EDTA  ethylenediaminetetraacetic acid

Terms

ABC   ATP-binding cassette transporters
AT    arabidopsis thaliana
ADP   Adenosine diphosphate
ARF   Auxin Response Factor
CAPS  Cleaved Amplified Polymorphic Sequence
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<td>PCR</td>
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<td>SSLP</td>
<td>Simple sequence length polymorphism</td>
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<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<td>TAIR</td>
<td>The Arabidopsis Information Resource</td>
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<td>GM</td>
<td>Ground Meristem</td>
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INTRODUCTION

Auxin is a unique signaling molecule controlling many plant development processes (Friml and Palme, 2002, Palme and Galweiler, 1999). This signal is perceived by cells and rapidly transduced into a wide variety of responses in growth and development including tropism (Rosen et al., 1999, Friml et al., 2002), patterning of early embryos (Jurgens, 2001, Friml et al., 2003), root patterning and elongation (Blilou et al., 2005), lateral root initiation (Casimiro et al., 2001), the positioning and expansion of leaves and flowers (Berleth and Sachs, 2001, Benkova et al., 2003, Reinhardt et al., 2003), and vascular differentiation (Berleth et al., 2000, Aloni, 2003). Polar auxin transport represents a special delivery system used by the plant to mobilize auxin from its site of synthesis in the shoots to basal sink tissues such as roots (Bennett et al., 1996). The control of auxin distribution is sufficiently precise to mediate differential cell behavior even within a small group of cells (Berleth and Mattsson, 2000). Multi-level feedback loops between the signal transduction network and the auxin transport network provide self-stabilizing patterns that remain sensitive to the external environment and to the developmental progression of the plant (Leyser, 2006).

Auxin enters the plant cell both by diffusion and through the facilitating action of an auxin influx carrier thought to be encoded by AUX1. Auxin cannot diffuse out of the plant cell and thus exits only through an efflux carrier apparatus that may involve the activity of at least two polypeptides, members of the PIN-FORMED (PIN) family (Palme and Galweiler), and members of the multidrug resistance P-glycoprotein (MDR/PGP) subfamily of ABC transporters (Muday and DeLong, 2001). Conditional gain-of-function alleles and quantitative measurements of auxin accumulation in Arabidopsis and tobacco-
cultured cells revealed that the action of PINs in auxin efflux is specific to auxin and sensitive to auxin transport inhibition (Petrasek et al., 2006). This suggests that PINs have direct involvement in catalyzing cellular auxin efflux. Both the AUX1 and PIN proteins show an asymmetric localization in the plasma membrane and the asymmetric localization of these membrane proteins underpins the characteristic polarity of auxin transport (Blilou et al., 2005).

Asymmetric PIN localization is dependent upon the actin-dependent cycling of vesicles through a mechanism similar to mammalian insulin-inducible GLUT4 glucose transporter system (Muday and Murphy, 2002). Sterols are required for correct docking to take place at the plasma membrane (Geldner et al., 2004). Plants mutant for PINOID (PID) also show similar phenotypes to pin1 because of mislocalization of PIN proteins. PID encodes a Serine Thr protein kinase and is known to control localization of PIN in an auxin dependent manner (Christensen et al., 2000). Loss-of-function and over expression of the PID gene has induced basal and apical placement of PIN proteins, respectively (Friml et al., 2004). Polar PIN localization and thus auxin efflux activity mediated by vesicle trafficking depends on the presence of functional GNOM (GN/EMB30, an exchange factor for adenosyl ribosylation factor GTPase-Guanosine exchange factor, ARF-GEF) which is functionally homologous to a yeast protein required for vesicle cycling and is sensitive to the fungal metabolite brefeldin A, (BFA). The membrane trafficking inhibitor BFA inhibits GN/EMB30 activity and results in PIN1 accumulation in endosomes (Muday and Murphy 2002).

In addition to the PIN and AUX1 proteins, members of the multidrug resistance P-glycoprotein (MDR/PGP) subfamily of ABC transporters have been shown to function in
the transport of auxin in both monocots and dicots. A link to auxin transport was suggested when hypocotyls of *AtPGP1* over expression transformants were found to elongate under dim light in a very similar manner to wild-type seedlings treated with low concentrations of auxin, and AtPGP anti-sense lines exhibited reduced elongation resembling seedlings treated with auxin transport inhibitors like 1-*N*-naphthylphthalamic acid (NPA) (Sidler et al., 1998). Subsequently, interruption of the gene encoding AtPGP19/AtMDR1, an auxin-inducible close homologue of AtPGP1, was found to result in partial dwarfism and reduced polar auxin transport (PAT) in hypocotyls and inflorescences (Noh et al., 2001).

It has been suggested that PIN–PGP pairings provide specificity and directionality to polar auxin transport (Luschnig, 2002). Comparative analysis of developmental phenotypes of *pin* and *pgp* mutants suggests that both PINs and PGPs function as transporters or transport activators in some way, with PIN proteins providing a basal transport vector and PGPs providing increased cellular loading and unloading (Geisler and Murphy, 2006). Of the 21 genes encoding the PGP subfamily of ABC transporter super-family only three *Arabidopsis* PGPs (PGP1, PGP19, and PGP4), all of which have been shown to bind to the auxin efflux inhibitor 1-*N*-naphthylphthalamic acid (NPA) with high affinity, have been biochemically characterized (Geisler and Murphy, 2006). PGP1 and PGP19 showed auxin efflux activities in plant, yeast, and animal cells (Geisler et al., 2005, Petrasek et al., 2006, Blakeslee et al., 2007). Co-expression of PGP4 and PIN2 resulted in enhanced uptake while PGP4 co-expression with PIN1 reversed PGP4 uptake transport suggesting that specific tissues regulate auxin transport in animal systems (Blakeslee et al., 2007). While this study suggests that PGP4 acts as an auxin influx
protein, a recent study by Cho et al., 2007 in two independent plant cell systems, the *Arabidopsis* root hair cells and tobacco cells, showed that *Arabidopsis* PGP4 displays auxin efflux transporter properties.

Once auxin is transported to a cell, auxin response in a cell is mediated through the ubiquitin degradation pathway. The pathway involves ubiquitin-mediated proteolysis of a set of transcription factors, Aux/IAA proteins (Leyser, 2001). Auxin Response Factors (ARFs) bind to Auxin Response Elements (AREs) and either activate or repress transcription. The activity of ARF is modulated through heterodimerization with AUX/IAA proteins that act as transcriptional repressors. Increased auxin causes phosphorylation of Aux/IAAs marking them for proteolysis, and releasing the ARFs to alter transcription of target genes. As the Aux/IAA gene promoters contain AREs that are themselves responsive to auxin induced ARF activity, the AUX/IAA-ARF proteins are components of a potential auto-feedback loop that may be important in responses to auxin.

Numerous experiments suggest that auxins, such as indole-3-acetic acid (IAA), can contribute to vascularization (Berleth et al., 2000a). Local application of auxin induces the formation of vascular strands (Sachs 1981) and high levels have been detected in pre-procambial cells (Mattsson et al., 2003). This alone is enough to predict that auxin acts as a positional cue for vascular development. Yet the specific mechanism by which auxin forms complex and unique venation patterns in leaves is poorly understood.

In *Arabidopsis*, leaf vein patterning is a progressive and hierarchical process. Ground cells are progressively recruited into vascular cell fate, which eventually form
veins in a spatially regulated manner. Vascular tissues are derived from elongated precursor cells called procambium, which form from the undifferentiated ground meristem (GM) cell population and later differentiate into vascular elements (xylem and phloem) (Scarpella et al., 2004). As the leaf primordium forms, parallel formation of the midvein is in continuation with the central axis of the plant and connects with the vascular tissue of the stem. As the lamina expands, the secondary veins diverge from the midvein and tertiary veins from secondary veins. The secondary veins connect to one another or to higher order veins at the distal margins to form closed areoles (Turner and Sieburth, 2002, Steynen and Schultz, 2003).

Of the several hypotheses proposed to explain vein pattern formation, Sachs (1981) proposed a mechanism, the auxin signal flow canalization hypothesis, that produces the continuous strand formation and likely the final vascular pattern. The auxin canalization hypothesis states that a positive feedback mechanism causes auxin-transporting cells to become more efficient in auxin flux (both influx and efflux) resulting in stable ‘auxin canals’. The increased conductivity of these cells would not only lead to their vascular differentiation (caused by the high levels of auxin), but would also deplete neighboring cells of auxin preventing them from taking on a vascular cell fate (Sachs, 1981).

Auxin biosynthesis occurs in both aerial portions of the plant and in roots. In early developmental stages, cotyledon-derived auxin has been suggested to be exported acropetally into the shoot apical meristem and the first leaf primordium (Cnops et al., 2006). Basipetal transport into this new primordium results in the formation of the midvein (Mattson et al., 1999. Sieburth, 1999, Aloni, 2001, Avsian-Kretchmer et al.,
Slightly later in development as lateral growth occurs, the primorium is transferred from a sink to a source of auxin, first at the leaf tip and then at the margins (hydathodes). This coincides with formation of the secondary veins, starting apically from the midvein (Avsian-Kretchmer et al., 2002). At a later stage, free auxin is also produced at low levels in the leaf lamina, inducing the tertiary and quaternary veins (Aloni et al., 2003). Recent studies have uncovered that auxin is also synthesized to some extent in roots, with the most prominent auxin source located in the meristematic zone of primary root tip and developing lateral roots (Ljung et al., 2005). Thus, the auxin required for root development could come from shoot and/or root source.

A vast majority of studies support the model that polar auxin transport plays a central role in vascular pattern formation (Nelson and Dangler 1997, Berleth et al., 2000, Aloni 2001, Scarpella et al., 2006). The PIN1 protein is the earliest detected pre-procambial marker in incipient vein cells and its subcellular localization in the cells contribute to procambium formation for all vein classes (Scarpella et al., 2006). The PIN polarity gives directionality to the auxin flow and the auxin flow determines the selection of pre-procambial cells, consistent with canalization concept. Analysis of vascular development in plants in which auxin transport is defective either because of mutation in PIN1 or because the plant was treated with a chemical transport inhibitor (Mattsson et al., 1999, Sieburth et al., 1999, Mattsson et al., 2003) support the canalization hypothesis. Plants mutant for PIN show increased leaf marginal venation (Mattsson et al., 1999). The effect of auxin transport inhibition (Mattsson et al., 2003) indicates a role for auxin signals in restricting vascular differentiation to narrow zones, promoting vascular continuity, and specifying the venation pattern in leaves. Compared to control plants, first
leaves of treated plants exhibited increased vascularization at the margin near the distal tip and increased vasculature in the petiole and midvein region due to an increase in number and size of vascular bundles. As suggested by DR5::GUS expression in the leaves, vascular differentiation occurs at sites of maximum auxin response and proper positioning of these auxin response maxima requires polar auxin transport (Mattsson et al., 2003).

The dynamics of PIN::GFP localization have clarified how auxin transport defines vein pattern (Scarpella et al., 2006). As suggested by PIN::GFP expression, at the earliest stage, PIN1 is expressed asymmetrically towards the apex in the marginal epidermal cells of leaf primordia and creates a convergence point at the apex which then directs the midvein basipetally into the internal cells (Scarpella et al., 2006). In generating loops of secondary veins, a convergence point is established at the leaf margin, and PIN is then expressed in underlying cells asymmetrically at the ends facing the midvein. The auxin then moves from the marginal source to the sink (midvein) connecting a linear file of cells (pre-procambium) to the existing strand. If this linearity is arrested a freely ending veinlet is formed (Scarpella et al., 2006). Generally higher order veins are free ending in wild type, however mutants that affect PIN localization such as cvp2-1, fkd1, sfc40 (Carland and Nelson, 2004, Deyholos et al., 2000, Hou et al., manuscript in preparation) exhibit more than normal frequency of non-meeting veins in secondary as well as higher order veins.

Given the importance of auxin in vascular development, auxin response mutants should also have profound effects on the development of vasculature. The auxin response gene AXR1 contributes to auxin response by targeting AUX/IAA proteins for degradation
through the ubiquitin pathway (Berleth et al., 2000a). AXR1 has been shown to be expressed in the vasculature of leaves and all other organs based on in-situ hybridization, AXR1::GUS expression and immunolocalization (del Pozo et al., 2002). axr1-3 has significantly fewer aeroles and branch points compared to wild type (Steynen and Schultz, 2003). Impaired auxin response due to mutation in MONOPTEROS (MP) (Przemec et al., 1996) is characterized by severe defects in the vascular system, embryo axis formation and consequent seedling lethality. MP is member of ARF family that functions to activate expression of auxin inducible genes important in producing an auxin response (Hardtke and Berleth, 1998). The mp partial loss of function mutants show a reduction in vasculature in the cotyledons, leaves and other organs. Complete loss of MP function results in loss of roots and shoot, and decreased vasculature in the cotyledons.

Shoot branches arise from axillary shoot meristems, located in the axils of a leaf and their outgrowth depends in part on leaf-derived auxin transported basipetally through the shoot (Ward and Leyser 2004). Both auxin and cytokinin have a major influence on bud outgrowth. Basipetally transported auxin inhibits bud outgrowth (Leyser, 2006), whereas cytokinins travel acropetally and promote bud outgrowth (Ongaro and Leyser, 2008). The highly branched shoot phenotype of mature axr1 mutant plants has been taken as genetic evidence for a role of auxin in the control of shoot branching (Stirnberg et al., 1999). With respect to the regulation of lateral bud outgrowth, several gene products (MAX1-MAX4) have been identified that act in the synthesis, perception and transduction of an unknown branching inhibiting molecule (McSteen and Leyser, 2005). Sorefan et al. (2003) showed that mutations in MAX4 gene of Arabidopsis result in increased branching and auxin resistant bud outgrowth, and that MAX4 acts downstream.
of auxin to produce a mobile branch-inhibiting signal. A similar role has been proposed for the pea gene, \textit{RMS1} (Beveridge et al., 2000 in Sorefan et al., 2003).

Auxin also contributes to the patterning of the primary root (Friml et al., 2004) and the formation of organs such as the lateral roots and root hairs (Benkova et al., 2003). Considerable progress has been made in both proving the essential role of auxin in root tip patterning, and the mechanism behind this process. The apical tip is the meristem or zone of cell division. The next zone proximal to the meristem is the zone of elongation where cell division ceases and there is rapid cell growth by elongation. Then comes the zone of differentiation or specialization, where cells assume their final fate (Dolan et al., 1993). The auxin level in root involves three basic mechanisms: a transport-regulated auxin gradient in the root meristem (Sabatini et al., 1999), local auxin biosynthesis and catabolism contributing to the auxin concentration profile (Ljung et al., 2002), and SFC TIR1 mediated proteolysis regulating auxin response in different root hair zones (Estelle, 2004). In roots, there are two polarities of auxin movement that are linked to different physiological processes. Auxin entering the root from the shoot is acropetally transported through the central tissues of the root toward the tip, where it is presumably combined with apically produced auxin (Ljung et al., 2005), redistributed toward the flanks, and then transported basipetally through the lateral root cap and epidermis (Swarup and Bennett, 2003). Both of these polarities of IAA movement have been detected and linked to specific physiological processes (Reed et al., 1998, Rashotte et al., 2000).

Inhibition of polar auxin transport and/or improper auxin flux affects the distal auxin maximum, which correlates with the pattern formation, orientation and extent of cell division (Sabatini et al., 1999). The gravitropic bending of root has been suggested
to be mediated by redistribution of auxin from the normal polar transport stream to lateral
transport across the root (Muday and Delong, 2001). Auxin is redistributed in response to
gravity so that it accumulates along the lower side of the root tip (Young et al., 1990).
This redistribution of auxin by PIN3 in the root tip is suggested to be important for
gravitropism because removal of the root tip abolishes the response (Blancaflor et al.,
1998, Friml et al., 2002). Both mutants resistant to auxin, axr1 and auxin transport
mutants (eir, aux1-7 and pin3) are agravitropic supporting the involvement of auxin in
gravitropism. (Lusching et al., 1998, Lincoln et al., 1990, Marchant et al., 1999, Friml et
al., 2002). The finding that NPA arrests root elongation suggests that auxin transport is
also necessary for root elongation (Muday, 2001). The role of another phytohormone
gibberellin has also been associated with root elongation and it has been suggested that
auxin controls the growth of roots by modulating cellular responses to gibberellin (Fu and
Harberd, 2003).

Lateral roots (LR) initiation is also controlled by auxin. LR initiate from internal
cells of the pericycle in the late cell elongation/early cell differentiation zone, in
pericycle cells that are partially to fully differentiated. Later in the development of the
root, these cells can undergo a defined program of oriented cell divisions and expansion
to form a lateral root primordium (Malamy and Benfey, 1997, Dubrovsky et al., 2001,
Casimiro et al., 2003). Extensive proliferation of adventitious and lateral roots develop in
plants with elevated auxin content like rty. The RTY/SUR1 gene encodes a protein that is
similar to Tyr aminotransferases and that is possibly implicated in auxin synthesis
(Golparaj et al., 1996). The RTY gene product has a critical role in regulating auxin
concentrations and thereby facilitating normal plant growth and development. The mutant
phenotype is due to accumulation of increased free IAA (Boerjan et al., 1995, King et al., 1995). Conversely mutants with reduced sensitivity to auxin such as \textit{axr}-1 and \textit{axr}-2 produce fewer LR than wild type (Estelle and Somerville, 1987). The \textit{axr}1-3 mutant produces about 70% fewer LRs compared to wild type and no lateral roots when in combination with another auxin resistant mutant, \textit{axr}4 (Hobbie and Estelle, 1995). The more extreme \textit{axr}1-12 produces no lateral roots by inhibiting initiation, as indicated by a lack of pericycle CYCB1:GUS expression (del Pozo et al., 2002). Incorporating the AXR1:GUS reporter in wild-type plants revealed that \textit{AXR}1 is expressed in all actively dividing cells of the root and shoot, including presumptive LR primordium pericycle cells and LR primordium (del Pozo et al., 2002).

Along with auxin levels, auxin transport has also been implicated in lateral root emergence and development. Both auxin influx and auxin efflux mutants have been shown to influence lateral root initiation, providing some insight into how the localized auxin maximum is created. In the loss of function auxin influx mutant \textit{aux}1, about half of the wild type number of LRs are produced (Marchant, et al., 2002). The AUX1 protein is important in shoot derived auxin (acropetal) pulse which has been suggested to promote the emergence of lateral root primordia (Bhalerao et al., 2002). Casimiro et al., 2001, from their study on the \textit{shoot meristemless} 1 mutant have shown that basipetal and acropetal polar auxin transport activities are required during the initiation and emergence phases respectively. A direct involvement of \textit{AtPGP}4 in auxin transport processes controlling lateral roots was suggested since \textit{AtPGP}4 loss of function enhanced lateral root initiation (Santelia et al., 2005). Root hairs are an excellent model system to study hormonal signals in cell developmental processes. They are easily accessible, single-
celled, long tubular extensions of root epidermal cells formed in the differentiating zone of the root (Ringli et al., 2005). The trichoblasts (hair-forming cells) and atrichoblasts (non-hair cells) are arranged in alternating files along the root surface so hairs are produced in a simple and invariant striped pattern (Dolan et al., 1994, Dolan and Costa, 2001). Genetic and physiological studies implicate auxin and ethylene in root hair development (Pitts et al., 1998). Several mutants defective in auxin transport show alterations in root hair growth that are most likely due to either a defect in auxin supply to the root hair cell or a loss of auxin transporting activity in the hair cell (Cho et al., 2007), which in either case changes intracellular auxin levels. The root hair model for cellular auxin transport relies on the simple fact that auxin transporter-mediated changes in intracellular auxin levels can influence root hair elongation: shorter root hairs by enhanced auxin efflux (or reduced influx) activity, and longer root hairs by enhanced auxin influx (or reduced efflux) activity (Cho et al., 2007). Overexpression of PIN3 (the auxin efflux transporter) or PINOID (the positive regulator of PINs) in the root hair cell greatly reduced root hair length (Lee and Cho, 2006). PGP4, was also strongly expressed in the root hair cells and plants mutants for PGP4 showed enhanced root hair elongation suggesting that PGP4 catalyzes auxin efflux in the root hair cells. Further, when PGP4 was overexpressed, it inhibited root hair elongation (Cho et al., 2007). Root hair–specific overexpression of PGP1 and PGP19, members of the same family of ABC transporters (Geisler et al., 2005, Petrasek et al., 2006) also decreased root hair elongation, further supporting the role of auxin transport in root hair elongation.

In this thesis, I describe the Arabidopsis mutant grassy (gsy) which, my analysis indicate, has reduced vein complexity and vein meeting, and increased growth of lateral
buds, decreased internode elongation, reduction in lateral root and root elongation, as
well as increased root hair growth. The spectrum of phenotypic defects is consistent with
defects to auxin and here I propose that \textit{GSY} is likely a component of the global
mechanism that controls auxin transport.
MATERIALS AND METHODS

Plant Materials

*Arabidopsis thaliana*, Columbia ecotype, was used as a wild type control in all experiments. Lines of *Arabidopsis* previously treated with ethyl methanesulfonate (EMS) were purchased from Lehle Seed (Round Rock, TX). Glabrous (*gl1-1*), *axr1-3* seed of Columbia ecotype (Col) was generously provided by George Haughn (Department of Botany, University of British Columbia, Vancouver, BC), *pin1-1, mp^{G92}* by Thomas Berleth (University of Toronto, Toronto, ON), *cvp2* by Francine Carland and Timothy Nelson (Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT) and *DR5::GUS* by Jane Murfett (University of Missouri, Columbia, MO). *FKD1::GUS* seeds were produced in the lab (Hou et al., manuscript under preparation). All other seed material (*aux1-7, eir1/pin2* and *rty*) was obtained from the *Arabidopsis* Biological Resource Center (Columbus, Ohio).

Growth Conditions

Seed were planted either on a mixture of ¾ Flora Compo compost (The Professional Gardener Co Ltd., Calgary AB) and ¼ vermiculite (Coaldale nurseries, Coaldale, AB) in 100 cm² pots or on Petri dishes containing *Arabidopsis thaliana* (AT) growth medium (Ruegger et al., 1998). Pots were covered with saran wrap and both pots and dishes were incubated at 4°C in the dark for 3 days, after which they were transferred to growth chambers (Percival Scientific, Perry, IA) with 24 hours of light at an intensity of approx. 130 μmol s⁻¹ m⁻² from Sylvania Cool White, Grow Lux, and incandescent bulbs (Osram Sylvania Inc, Danvers, MA). Chambers were set at 21°C and 60% relative
humidity. The day of transfer to the growth chambers was considered to be the day of germination or 0 Days After Germination (DAG). Saran wrap was removed at 7 DAG and plants were maintained under constant growth conditions.

**Mutant Isolation**

Approximately 6000 EMS (Kim et al., 2005) mutagenized M2 generation seeds in Columbia background (Lehle seed, Round Rock, TX) were sown at a density of about 30 seeds per pot and screened for aberrations in venation patterning at 14 DAG for cotyledons and 21 DAG for first rosette leaves. Putative vascular pattern mutants were grown to maturity and M3 seed was harvested and subsequently re-screened. The gsy mutant represents one of several mutants identified. The gsy mutant line was backcrossed to Columbia four times prior to all analyses.

**Mapping of gsy**

Mapping was carried out through the use of ecotypic specific markers visible through PCR (SSLPs) or a combination of PCR and restriction endonucleases (CAPS) as described in Lukowitz et al. (2000). gsy was crossed into the Landsberg (Ler) background, and DNA was extracted (after Dellaporta et al., 1983) from the leaves of F2 plants showing the gsy mutant phenotype to confirm homozygosity for gsy allele. F3 seed was collected from each plant. DNA from 30 F2 gsy plants was first used to map GSY to chromosome 1 using chromosome specific primer sets. SSLPs and SNPs (for CAPS) between Col and Ler in the flanking regions of chromosome 1 (Table 1) were identified through the Cereon polymorphism database (http://www.arabidopsis.org/Cereon/) (Jander
et al., 2002). The web-based programs Primer3 (http://frodo.wi.mit.edu/) (Rozen & Skaletsky, 2000), Blastdigester (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_blast_digest.cgi) (Illic et al., 2005), and dCAPS finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) (Neff et al., 2002) were used in locating or designing primers around each polymorphism. Generally large SSLP’s were used first, then SNPs (CAPS and dCAPS) were used in regions where SSLP’s could not be found. 380 gsy F2 plants were tested for segregation and PCR was done using standard conditions (Bell and Ecker, 1994). PCR products were resolved by gel electrophoresis using a 4% agarose gel at 100 or 150 V.

Ethidium bromide was purchased from Sigma Chemical Co (St. Louis, MO); dNTP’s were purchased from Invitrogen (Burlington, ON); primers were synthesized by Integrated DNA Technologies (Coralville, IA); Taq DNA Polymerase was purchased from New England Biolabs (NEB, Ipswich, MA); Restriction enzymes were obtained through NEB. Agarose was purchased from Bioshop Canada Inc. (Burlington ON).

**First leaf analysis of gsy**

To analyze and compare the first leaves of all genotypes, seeds were sown at a density of 20 seeds per pot. First leaves (21 DAG) of all genotypes were mounted in cytoseal or cleared overnight in 70% ethanol (V/V) and transferred to chloral hydrate (Sigma):water:glycerol (8:2:1 v/v/v, after Koizumi et al., 2000). For developmental series analysis, first leaves from wild type (Col ecotype) and gsy seedlings grown on AT medium were removed at 3, 4, 5, 6, and 7 DAG and treated as above in ethanol and chloral hydrate.
To examine the effects of NPA on leaf vascular development, *gsy* and wild type seeds were sown on AT dishes and AT dishes supplemented with 0μM, 5μM, 10μM, 30μM, 100μM NPA (Chem Service, West Chester, PA) concentrations. These chemicals were added to the autoclaved medium. The leaf phenotype was examined at 10 DAG.

**Plant shoot morphology**

To analyze plant shoot morphology, *gsy* and Col seed were sown on soil at a density of 25 seeds per pot. Final density was maintained at 12 healthy plants per pot from two leaved stage. Plants were scored for days to bolting of 50% of plants, leaves at bolting and at 35 DAG for number of rosette leaves, rosette and cauline branches and internode length.

**Root assays**

To study the effects of the synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) on root length and lateral root formation, seeds were sown on non-supplemented AT medium and the seedlings were transferred to supplemented media (medium containing either 1.0x10^{-9}, 1.0x10^{-8} or 1.0x10^{-7} M 2,4-D). Root growth was measured from the position of the root tip at transfer to the position 4 days later (Steynen and Schultz, 2003). To analyze the gravitropic response of roots, seedlings were grown vertically on petri dishes containing AT medium for 5 days. Plates were then rotated 90° and the gravitropic response was measured after 72 hours.
Generation of Double Mutants

Double mutants were generated between gsy and \( mp^{G92}, pin1-1, axr2, axr1-3, eir1, cvp2-1, rty1, \) and \( aux1-7. \) All double mutant populations were screened in the F2 generation and ratios were analyzed using the chi-square goodness of fit statistic. Seeds from the F2 plants that were homozygous for gsy were harvested. When the double mutant plants were fertile, double mutants segregating in the F3 were allowed to self fertilize and F4 plants were characterized. If double mutants were sterile (\( pin1, rty, mp \)), analysis was done in the segregating F3 population. In generating double mutant lines with \( eir1, \) and \( aux1-7, \) lack of gravitropism was used to confirm the presence of the other mutation in the double mutant. In case of \( axr1-3, \) resistance to \( 1\times10^{-7} \)M 2,4-D was used in AT medium to isolate and confirm the double mutant.

All the double mutants, except for \( gsy mp, \) were analysed for 21DAG first leaf, root hairs, shoot and root branching as well as root gravitropic assays. Leaves were scored for total number of secondary veins and number of non meeting secondaries (NMS), total number of tertiaries and number of non meeting tertiaries (NMT), total number of quaternary veins, number of areoles and number of vascular islands. Since \( gsy \) \( mp \) failed to produce proper leaves, shoot and root; only 14-day old cotyledons could be characterized. Cotyledons were scored for number of secondary veins and number of areoles.

In this paper, the midvein (primary vein) is considered to be the linear vascular strand approximately along the midline, secondaries are considered to be those vascular strands connected to the midvein, tertiaries are veins connected to secondaries (but not the midvein), and quaternaries are veins connected to tertiaries (but not the midvein or...
secondaries). These vascular strands were identified based on differentiated xylem. Areoles (area of leaf completely enclosed by veins), vascular islands (fragments of discontiguous vasculature), as well as free-ending secondary and tertiary veins (veins connected at one end but disconnected at the other end were scored.

**Histochemical staining for GUS**

The gsy mutation was introduced into *DR5::GUS* (Ulmasov et al., 1997) transgenic plants by out crossing of gsy plants with homozygous *DR5::GUS* plants in the Col-0 background. Plants expressing gsy phenotype in the F2 were allowed to set seeds. Individual F3 populations that segregated for *DR5::GUS* were allowed to self fertilize, and a homozygous F4 generation was used for characterization. A similar procedure was used for generating a gsy *FKD1::GUS* line (Hou et al., manuscript under preparation)

Seed from the gsy *DR5::GUS*, and Col *DR5::GUS* lines were planted on AT plates with or without chemical treatments. Seedlings were stained for 6 hours. GUS staining were performed after Kang and Dengler (2002). Seedlings were removed from the medium, kept under chilled condition of acetone for 20 minutes and washed twice with 50μM sodium phosphate buffer pH 7 wash solution. The seedling were stained with GUS staining buffer [5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), Rose Scientific, Edmonton, AB] followed by vacuum filtration for 10 minutes and then left for 6 hours (unless otherwise stated) before the GUS buffer was removed and decolourized with four rinses of 70% ethanol and finally cleared with chloral hydrate.
**Microscopy, Imaging and Statistics**

A Leica MZ8 dissecting light microscope (Leica Microsystems, Wetzlar, Germany) was used for analysis of mature cotyledons, leaves and flowers. Seedlings were dissected by hand using 23 gauge needles (Becton Dickinson, Oakville ON) and were mounted on slides with 50% glycerol. Analysis of leaf developmental stages, *FKD1::GUS* and *DR5::GUS* expression in leaves, and auxin transport inhibitor treated leaves was performed using an Eclipse E600 compound light microscope (Nikon, Mississauga, ON). Tissues were photographed using a Nikon Coolpix 990 camera (Nikon, Mississauga, ON) and analyzed using Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA) and NIH Image (http://rsb.info.nih.gov/nih-image/). Measurements were recorded in Microsoft (Redmond, WA) Excel for subsequent determination of averages, standard errors, and P values by F-test and Student's t tests. Data sets, which had significantly different variances (p<0.05), as determined by the F-test, were analyzed using two tailed T-test assuming unequal variances, otherwise two tailed T-test assuming equal variances was used.
RESULTS

We have identified a leaf vein-patterning mutant generated through EMS mutagenesis of *Arabidopsis thaliana* seeds (Columbia ecotype, Col-0). In addition to a simplified and non-meeting vein pattern, the plants mutant for GRASSY (*GSY*) show distinct morphological characteristics that include defects to root and shoot morphology suggesting *GSY* has a global role in plant development.

Mutant isolation and Mapping of *GSY*

An M2 population of *Arabidopsis* (*gl1-1, Col background*) previously treated with ethyl methanesulphonate (EMS) was screened for vein pattern defects using Columbia ecotype as a control for phenotypic comparisons. From this initial screen, a leaf vein patterning mutant (isolation family 32-14-1) showing simple first leaf vein pattern with reduced numbers of secondary, tertiary and quaternary veins was identified. Furthermore, secondaries as well as higher order veins are often non-meeting and form somewhat parallel to one another. Finally the leaves are narrow and pointed so that the overall leaf phenotype is reminiscent of grass leaves; hence we named the mutant *grassy* abbreviated hereafter as *gsy*.

Polymorphism such as simple sequence length polymorphism (SSLP) and Cleaved Amplified Polymorphic Sequences (CAPS) have been identified and compiled for two *Arabidopsis* ecotypes, Columbia (Col) and Landsberg erecta (*Ler*) in Monsanto (http://www.arabidopsis.org/browse/Cereon/help.jsp). In order to map the *GSY* gene, *gsy* (Columbia background) mutant was crossed to Landsberg erecta (*Ler*) and the
segregating F2 population was used for molecular mapping (Bell and Ecker, 1994). Several new CAPS and SSLP markers in chromosome 1 were developed from the TAIR database (Table 1). From a total of 760 chromosomes (380 F2 gsy samples) GSY was mapped (Figure 1) to a 135 KB region flanked by 1-42-C and the nga392 marker on chromosome 1. This corresponds to two adjoining Bacterial Artificial Chromosome (BAC) clones (F28L5 and F13K9) and includes 28 putative gene loci (Table 2).

**gsy has simplified and non-meeting first leaf phenotype**

In order to determine the extent to which GSY is required for the development of vascular pattern, 14 Days After Germination (DAG) cotyledons and 21 DAG first leaves of gsy mutant and the Col-0 Wild Type (WT) accession were dissected and cleared in cytoseal to analyze for vein pattern defects (Figure 2). The gsy cotyledons were not different from WT in terms of gross morphology and vein pattern except that they were smaller in size (Figure 2B, Table 3).

In wild type first leaf, the midvein (primary vein) runs proximodistally along the midline in continuation with the stem vasculature. Distal secondaries are initiated and connected to the midvein. Tertiaries are connected at least at one end to secondaries and quaternaries to tertiaries forming areoles (regions of the lamina completely enclosed by veins). (Figure 2A a, Steynen and Schultz, 2003, Kang and Dangler, 2004, Scarpella et al., 2004).

In order to determine the specific quantitative differences between gsy and wild type first leaves, several vascular patterning traits, namely secondary veins, tertiary veins, quaternary veins, areoles, non-meeting secondary and tertiary veins as well as vascular
islands (fragments of discontinuous vasculature), were compared. Two classes of venation complexity are observed in *gsy* first leaves the average of which is quantified in Table 4. Leaves of the first class showed a complex venation pattern similar to wild type but with increased numbers of areoles formed by tertiary veins adjacent to the leaf margins (Figure 2Ac). These leaves very much resembled those of plants mutant for *pin1* (Figure 2Am) or leaves treated with low concentrations of an auxin transport inhibitor like NPA. Leaves of the second class (strong *gsy* phenotype) showed a reduction in vein numbers (secondary, tertiary and quaternary) and vein meeting. The increased number of free ending secondary veins combined with fewer vein numbers of all orders led to fewer areoles, which we referred to as simplified non meeting vein phenotype (Table 4, Figure 2A b). Though not statistically different from wild type which never has vascular islands, vascular islands are sometimes observed in *gsy*. Higher order rosette leaves of *gsy* are smaller and have more hydathodes than the WT leaves, but show no obvious differences in vein continuity and complexity and were not analyzed quantitatively.

To determine when the defects in *gsy* first rosette leaves start to appear, I compared wild type and *gsy* mutants for expression of *FKD1::GUS*, a marker that is expressed in procambium at a similar time as *AtHB8::GUS* and is therefore an early marker of vascular fate (Hou et al., manuscript in preparation) and for xylem lignification which corresponds to a late stage of vein formation. First leaves of 5, 6 and 7 DAG seedlings were dissected, cleared in chloral hydrate and scored under dark field optics for xylem lignification. Development of midvein, number of secondaries, tertiaries, areoles and non-meeting secondaries were analyzed for developmental delay (Table 5). Leaves of *gsy* are smaller at all stages. Vascular development is delayed from the development of
midvein to completion of tertiary veins. At 5 DAG, distal secondaries are fully formed in wild type but are not yet visible in gsy leaves (Figure 3d) and at 6 DAG, when proximal secondaries are complete in WT, distal secondaries forming one or two areoles are just formed in gsy. By day 7, when we expect most of the secondaries to have completely developed and form complete loops marginal venation gaps were evident in gsy (Table 5, Figure 3f). In wild type, the majority of secondary vein xylem lignification occurs between day 5 and day 6, whereas in gsy, the majority occurs between day 6 and day 7. Similarly tertiary vein xylem lignification is first evident in wild type at day 6, whereas it is evident in gsy at day 7. At 7 DAG FKD1::GUS expression also shows that there are regions of distal non meeting in gsy (Figure 3B). This suggests that GSY acts prior to procambial specification and xylem differentiation in leaf development.

**DR5:GUS expression is intact in leaves**

Auxin has been shown to play a critical role in vascular differentiation. The major hypothesis to explain vein patterning, canalization of auxin flow, proposes that vein position is specified by paths of high auxin flux (Sachs, 1981). A number of Arabidopsis mutants with open and/or simplified vein pattern including lop1/tornado1 (Carland and McHale, 1996), sfc (Dayholos et al., 2000), fkd1 (Steynen and Schultz, 2003, Hou et al., manuscript in preparation) and hve (Candela et al., 1999) have been characterized and found to be defective in either auxin transport or auxin response. Based on the correlation between defects in auxin transport or response and simplified vein pattern, we expect the vein pattern defects in gsy could arise due to either improper auxin transport or low level of response to the transported auxin.
To ascertain whether the reduced vein number of the gsy mutants results from reduced responsiveness to auxin, we studied the expression of the \textit{DR5::GUS} reporter construct (Ulmasov et al., 1997). The \textit{DR5::GUS} reporter line is a synthetic construct with 7 auxin response promoters in tandem coupled with B-glucuronidase (GUS) reporter gene (Ulmasov et al., 1997). This promoter-marker gene fusion is activated by auxins to visualize auxin response. Patterns of GUS staining conferred by DR5 in WT leaves have been well characterized (Aloni et al., 2003, Mattsson et al., 2003). Previous studies have shown that \textit{DR5::GUS} expression precedes and coincides with the appearance of procambial strands, and then disappears as the veins mature. In young \textit{Arabidopsis} leaf primordia, the expression of this marker gene predicts sites of vascular differentiation and local auxin levels suggesting that a local accumulation of auxin determines provascular and proprocambial cells (Mattsson et al., 2003).

To test if gsy leaves show altered auxin response, DR5:GUS was introduced into the gsy background, and the intensity and pattern of DR5:GUS expression were compared between WT and gsy first leaves (Figure 4). While the final pattern and intensity of expression are similar to wild type, each stage is delayed in gsy compared to wild type. In WT leaf primordia GUS expression is first observed in the distal leaf tip and then in midveins. With the expansion of leaf lamina and differentiation of midvein at 3 DAG, \textit{DR5::GUS} expression diminishes from the midvein and new expression zones appear at the site of future secondary vein loops, followed by new zones of expression, that coincide with the formation of tertiary veins (Figure 4). In contrast, in 3 DAG gsy first leaves, the expression is weak and limited to distal tips and it is only in 4 DAG leaves that high distal tip expression with loops predicting presumptive secondary veins
are visible (Figure 4g). Further as the development progresses, at 5 DAG, \textit{DR5::GUS} is expressed in wild type proximal tertiary veins, but in \textit{gsy} it is only at day 6 that we see similar stage. These observations are consistent with development in \textit{gsy} being delayed by about 1 day compared to WT. However, within the developing vein \textit{DR5::GUS} expression is often reduced in \textit{gsy} compared to wild type. By day 7 expressions in both genotypes is confined mainly to the hydathodes and distal areas and shows a little difference in intensity of expression suggesting that \textit{gsy} is capable of a similar level of auxin response as wild type. In wild type, the \textit{DR5::GUS} expression at 5-7 DAG can often be seen as forming discontinuous loops and in \textit{gsy} continuous loops are rarely seen and gaps at distal junction are frequent (Figure 4, asterisks). Thus the discontinuous \textit{DR5::GUS} expression pattern predicts the later vein non-meeting pattern. This suggests a lack of vascular cell fate in those regions either due to defective auxin response or auxin transport.

\textbf{gsy shoot morphology}

The defective first leaf phenotype of \textit{gsy} is consistent with altered auxin response or transport, and \textit{DR5::GUS} expression provides evidence that \textit{gsy} may be defective in auxin transport. Because auxin is responsible for controlling a whole range of shoot and root morphology and proper patterning of the whole plant, I examined additional auxin related defects in \textit{gsy}. Phenotypic analysis of \textit{gsy} and WT showed that \textit{GSY} has an important role in \textit{Arabidopsis} shoot and root patterning.

Auxin is primarily synthesized in young leaves and is transported down the plant body in a polar fashion (Lomax et al., 1995). Basipetal transport of auxin through the
plant body from its site of synthesis in young leaves inhibits shoot branching by inhibiting elongation of bud growth (Leyser, 2003). The \textit{axr1} mutation does not affect the timing of axillary meristem formation; however, subsequent lateral shoot development proceeds more rapidly in \textit{axr1} plants (Stirnberg et al., 1999). As well, auxin response is important in regulating flowering time and inflorescence height. Mutation in ARF2, a transcriptional repressor of the auxin response, results in late flowering and elongated inflorescence (Okusima et al., 2005). \textit{gsy} mutants have defects in plant growth at a variety of developmental stages. Compared to wild type, \textit{gsy} plants are dwarf and have fewer rosette and cauline leaves (Table 6, Figure 5). Less pronounced elongation in internodes of primary inflorescences, along with an enhanced initiation of axillary shoot in the rosette (Table 7, Figure 5b) confers a bushy and dwarf-like appearance to the adult mutant plant. The bushy appearance is intensified upon reiteration of bud outgrowth in secondary and tertiary inflorescence. \textit{gsy} mutants bolt, flower and produce mature seed earlier than wild type. They also have smaller siliques that shatter more easily than wild type.

\textbf{\textit{gsy} root phenotype}

Auxin is a key regulator of primary root elongation and gravitropism, lateral root development and root hair development (Laskowski et al., 1995, Estelle, 1996, Casimiro et al., 2001). While basipetal movement of IAA from the root tip back has been linked to root elongation and gravity response (Rashotte et al., 2000), basipetal and acropetal polar auxin transport activities are required during the initiation and emergence phases of lateral root development respectively (Casimiro et al., 2001). The primary root in \textit{gsy} was shorter and the growth rate was also significantly reduced compared to the wild type.
We also see a slight defect in root gravitropism where 6 DAG gsy roots, when grown vertically on AT media, showed a slight deviation from the normal vector (Figure 6). Plants mutant for GSY also showed significantly reduced number of lateral roots (Table 7). All of these changes suggest that basipetal transport may also be defective in gsy.

If gsy is defective in auxin transport then we might expect to see defects in root hair phenotype. Root hair elongation can be used as a biological marker to study the activity of auxin transporters (Cho et al., 2007). Several mutants defective in auxin transport show enhanced root hair length (Pitts., 1998, Rahman et al., 2002) that is most likely due to either a defect in auxin supply to the root hair cell or a loss of auxin-transporting activity in the hair cell (Cho et al., 2007). The gsy mutant root has longer root hairs than the wild-type root (Figure 7A). Enhancement of root hair elongation following the loss of GSY might imply that GSY catalyzes auxin efflux in root hair cells, resulting in increased auxin retention inside the hair cell and the stimulation of root hair elongation.

**DR5::GUS expression in roots.**

Inhibition of polar auxin transport and/or improper auxin flux affects the distal auxin maximum, which correlates with the pattern formation, orientation and extent of cell division (Sabatini et al., 1999). The role of the GSY in auxin transport was examined by analysis of the expression of DR5::GUS in the roots of wild type and the gsy mutant. Wild type plants displayed the highest GUS activity in the quiescent center, columella initials and mature columella root cap (Figure 8a,b, Sabatini et al., 1999). The GUS staining pattern in gsy (Figure 8 e,f,g) shows a range of variation in expression including stronger response in the regions including columella, root meristem and quiescent center.
(e), similar response but asymmetric in epidermal cells (f) and weaker expression (g). As well, \textit{gsy} often shows ectopic \textit{DR5::GUS} expression in the stele, lateral root cap and epidermis. The ectopic and asymmetric expression pattern, which is common in \textit{gsy}, was never observed in the wild type plants in identical conditions of growth media and staining duration. This suggests that \textit{GSY} is required for correct localization of the DR5 peak in the root tip. This mislocalization could also be linked to defects in root hair and lateral root suggesting that proper root patterning and cell division require proper auxin localization. Together with increased root hair length, reduction in lateral roots and slight defects of gravitropism, the ectopic auxin response in root epidermis is consistent with \textit{gsy} being defective in basipetal auxin transport.

**Effect of auxin transport inhibition in leaves and roots**

To further investigate the relationship between \textit{GSY} function and polar auxin transport in vein pattern formation, root patterning and overall morphology of the plant, I treated \textit{gsy} and WT plants with the auxin transport inhibitor, N-1-naphthylphthalamic acid (NPA). Treating root tissues directly with NPA arrests lateral root development by blocking the first transverse division(s) of xylem pole pericycle cells. NPA appears to exert its developmental effects by causing IAA to accumulate in the root apex while reducing levels in basal tissues critical for lateral root initiation (Casimiro et al., 2001). In \textit{gsy} the primary and lateral root growth was arrested at 1\(\mu\text{M}\) NPA compared to 10\(\mu\text{M}\) NPA in WT. Similarly, lack of gravitropic response and proliferation of root hairs was evident in \textit{gsy} at lower concentration (5\(\mu\text{M}\)) while in WT it was observed at 10\(\mu\text{M}\) (Figure 7B). Finally root tip bulging was also evident in \textit{gsy} at a lower concentration of
NPA (10μM) than in WT (100μM; Figure 9). This suggests that gsy is more sensitive to auxin transport inhibitors.

Using the DR5::GUS reporter gene, it has been shown that NPA could cause accumulation of free IAA in the root tips, leading to GUS staining of larger areas of root apex (Casimiro et al., 2001). I examined the effects of NPA on DR5::GUS-staining patterns in both wild type and the gsy mutants. The DR5::GUS expression domains in untreated wild type and the gsy mutant roots were essentially identical with respect to intensity. NPA treatment significantly increased the expression domains of DR5::GUS in both wild-type roots and gsy mutant roots (Figure 9). While the root length, root hair and lateral root data suggest gsy is defective in auxin transport and more sensitive to auxin transport inhibitors, the DR5::GUS expression upon NPA treatment would suggest that at the level of auxin response, treatment with NPA eliminates genotypic differences.

To further investigate the relationship between GSY function and polar auxin transport in vein pattern formation, I treated the gsy and WT plants with the auxin transport inhibitor, N-1-naphthylphthalamic acid (NPA). The transport of auxin from the leaf margin directs leaf venation patterns. When this transport is inhibited either chemically or genetically, DR5::GUS expression and vein proliferation occurs near the leaf margin, suggesting that the leaf margin is a major source of auxin (Mattsson et al., 2003).

WT and gsy plants homozygous for DR5:GUS were grown on various (0μM, 5μM, 10μM, 30μM and 100μM) concentrations of NPA in petri-plates. 10 DAG seedlings were stained, cleared and first leaves were dissected for the study. NPA treated wild type first leaves showed enhanced vascular differentiation along the entire lamina
margin, and the marginal vascular tissues were connected to the central vascular tissues with an increased number of non-branched vascular tissues (Figure 10). These effects depended on NPA concentration. In the first rosette leaves of wild type, midvein and marginal vein proliferation starts at 5μM and is subtle. The same phenomenon is already extreme in gsy at the same concentration of NPA treatment. This proliferation becomes increasingly more and more extreme in gsy until finally the whole leaf blade narrows and curls down with a parallel mass of midveins that extend from the margin proximally (Figure 10j). These results suggest that like gsy roots, gsy leaves are more sensitive to polar auxin transport inhibition.

**Exogenous auxin and root phenotypes**

I also examined root growth activity of gsy in the absence or presence of the exogenous auxin, 2,4-D. Resistance to the root growth inhibition caused by 2,4-D treatment is a standard assay for auxin resistance and auxin response mutants (Marchant et al., 1999). Exogenous auxin inhibits root elongation and stimulates lateral root formation (Casimiro et al., 2001). As shown in Table 8, on unsupplemented media, elongation of the gsy roots was significantly reduced compared to the corresponding wild-type plants. On growth media supplied with 10⁻⁹ μM or 10⁻⁸ μM 2,4-D growth rate of gsy mutant roots was significantly much reduced, compared to the respective wild-type plants (Table 8) suggesting that gsy is more sensitive to 2,4-D treatments. At the higher concentration 10⁻⁷ μM, the reduction in root growth was similar in both genotypes. This is consistent with auxin response being intact in gsy.
To examine whether GSY is required for auxin-mediated lateral-root formation, I examined the lateral-root formation in the presence or absence of exogenous auxin (Table 8). When grown vertically on unsupplemented media, gsy mutants produced fewer lateral roots than did wild type control plants (Table 7). When 5DAG seedlings were transferred to media supplemented with $10^{-8}$ M 2,4-D and grown for 4 days, gsy was sensitive to the auxin treatment and developed more lateral roots compared to wild type and at $10^{-7}$ M, the gsy mutant still produced the same frequency of lateral roots as wild type. Like the primary root growth assay, this suggests that gsy is more sensitive than wild type to low levels of 2,4-D and at higher level the auxin is in sufficient excess that genotypic differences are eliminated.

**Double mutants**

To investigate the functional relationship of GSY with genes required for auxin transport ($PIN$, $AUX$, $EIR$), perception ($AXR1$, $MP$) and synthesis ($RTY$) as well as a gene specifically required for vascular development ($CVP2$), I obtained the corresponding double mutants and quantitatively analyzed their venation patterns for secondaries, tertiaries, quarternaries, areoles, non meeting veins and vascular islands (Table 4), lateral root and shoot branching (Table 7) and root hair morphology (Figure 12).

**gsy and auxin transport mutants**

To examine possible roles of GSY in auxin transport we analyzed the double mutants of gsy together with $pin1$, $eir1$, $aux1$. The auxin efflux carrier PIN1 has been shown to be expressed at the earliest stages of leaf primordium development (Reinhardt et al., 2003 Scarpella et al., 2006). Like plants in which auxin transport has been inhibited chemically, plants homozygous for the loss-of-function allele, $pin1$-1, are defective in
polar auxin transport displaying phenotypes including increased marginal venation (Mattsson et al., 1999, Galweiler et al., 1998) and sometimes fused leaves. The overall leaf vein pattern of pin1-1 was not significantly different from wild type except that they had fewer non-meeting tertiaries. EIR/PIN2 is a root specific efflux protein with a less significant role in leaves. The roots are agravitropic and have a reduced sensitivity to ethylene (Lusching et al., 1998). No specific leaf vascular phenotype has been reported for the mutant and PIN2 transcripts were not found in 5 DAG leaves (Scarpella et al., 2006) but in this present study I found that the first leaf of eir is simpler with fewer tertiary and quaternary veins and fewer areoles. AUX1 encodes a membrane protein that is believed to be a component of the auxin influx carrier (Bennet et al., 1996, Merchant et al., 1999, Swarup et al., 2001). AUX1 regulates root gravitropism by facilitating auxin uptake within the root apical tissues (Marchant et al., 1999) so we expect the mutation in AUX1 to impair auxin influx carrier activity. Although no previous leaf vein defect has been reported (Steynen and Schultz, 2003) my analysis shows that like eir1, it is simpler with fewer veins and areoles.

Compared with gsy, the gsy pin1-1 double mutant first leaves were significantly more complex than gsy with increased numbers of areoles, secondary, tertiary and quaternary veins as well as fewer non-meeting veins (Table 4), suggesting that pin1 is epistatic to gsy in leaves. The pin1 mutants has wild type rosette leaves but a single pin-formed inflorescences with no cauline leaves, secondary inflorescence, flowers or siliques due to lack of auxin transported to the shoot apex from the young organs (Figure 5e). I also analyzed the effect of pin1 on gsy shoot branching which suggest that pin1 shoot phenotype is suppressed by gsy. The lack of organs in pin1 was rescued in gsy
background, both axillary shoots and partial flowers develop in gsy pin1. The suppression of pin1 by gsy in shoot apex suggest that gsy may work in opposition to pin1, perhaps by causing auxin accumulation in the shoot apex.

Like the gsy pin1 double mutant, the first leaf of gsy eir and gsy aux1-7 double mutants is more complex than gsy single mutant suggesting that gsy is suppressed (Table 4, Figure 2A). Other characteristics of gsy are also suppressed in other transport mutant backgrounds (Table 7). In gsy we have fewer lateral roots and higher number of rosettes branches whereas, in the double mutants with eir1-1 and aux1-7 the number of rosette branches is decreased and lateral roots increased (Table 7). This suppression of the gsy first leaf phenotype by eir1, aux1 or pin1 suggest either that gsy and the auxin transporters have opposing function or that the gsy phenotype results from over expression of these genes.

**gsy and auxin response (mp, axr1) and auxin synthesis (rty) mutant**

*AUXIN RESISTANT1 (AXR1)*, encodes a subunit of the RUB1-activating enzyme that promotes the modification of CUL1 with RUB1 of the ubiquitin pathway (Lincoln et al., 1990; Leyser et al., 1993, del Pozo et al., 2002) and loss of *AXR1* function causes auxin insensitivity and a bushy inflorescence, the latter due to a decrease in the inhibition of apical dominance mediated by auxin (Estelle and Somerville, 1987; Lincoln et al., 1990, Stirnberg et al., 1999). The axrl homozygous single mutant has a very simple first leaf venation with a higher number of non-meeting veins secondaries and lower numbers of secondary, tertiary, quaternary veins and areoles (Table 4, Figure 2A). The double mutant is more extreme than either of gsy or axr1 with higher number of non-meeting veins secondaries, fewer secondaries, terciaries, no quartenaries and very few areoles.
One could interpret the phenotype as either additive or synergistic suggesting that two genes function either in independent pathways or overlapping and partially redundant pathways. With respect to the shoot branching and root hairs, *axr1* appears to be epistatic to *gsy*, with the double mutant having higher number of cauline branches and sparsely distributed root hairs characteristic of *axr1* single mutant (Table 7, Figure 5).

The *MP* gene encodes an auxin response factor IAA24/ARF5 that mediates auxin signaling. Mutations in the MP gene interfere with the initiation of body axis in the early embryo and with the formation of vascular strands at all stages (Hardtke and Berleth 1998). Phenotypic characterizations have shown that loss-of-function allele *mp*<sup>G92</sup> mutants have no roots, highly reduced hypocotyls, and cotyledons with highly reduced vein pattern (Berleth and Jurgens, 1993). To examine the genetic interaction between the *gsy* and *mp*<sup>G92</sup> mutations, I generated *gsy mp*<sup>G92</sup> double mutants. Since no other organs except cotyledons were produced, I compared the cotyledons of the corresponding single and double mutant (Table 3). The cotyledons of *mp*<sup>G92</sup> mutants contained a midvein that rarely extends the full length and less often a secondary vein arising from the midvein that fails to join the midvein in the proximal region, thus no areoles (Figure 2B). The double mutant phenotype was essentially the same as *mp*<sup>G92</sup> single mutant in that it lacked hypocotyls, root and any leaves. The cotyledon vein formation was also similar to *mp*<sup>G92</sup> except that sometimes two secondaries originated from the midvein but again failed to form an areoles. The overall data suggest that that *mp* is epistatic to *gsy*. (Table 3).

To investigate further the role of GSY in auxin-regulated leaf root architecture, double mutants were made by crossing the *gsy* mutant with the *rooty* (*rty*) mutant. *rty* is
is characterized by high endogenous levels of auxin and the production of supernumerary lateral roots and adventitious roots on the hypocotyl (King et al., 1995, Boerjan et al., 1995, Figure 12m). The \textit{gsy \textit{rty}} double mutants produced the same root phenotype as the \textit{rty} mutant (Figure 12n). Again, like the strong adventitious root mutant \textit{rty} (Boerjan et al., 1995, King et al., 1995), \textit{gsy \textit{rty}} double mutant was also sterile. Finally, the first leaf of \textit{rty \textit{gsy}} showed drastic simplification in its vein pattern very similar to \textit{rty} and often resembling that of the cotyledon (Figure 2A\textit{j,o}). This overall result suggests that \textit{gsy} is sensitive to auxin. \textit{rty} is epistatic to \textit{gsy} and the simple vein pattern of \textit{gsy} cannot be ameliorated simply by increasing auxin levels.

\textit{gsy and cvp2}

CVP2 encodes an inositol polyphosphate 5’ phosphatase and the mutants produce first leaves and cotyledons with non-meeting veins veins and vascular islands (Table 4, Figure 2A\textit{d}, Carland and Nelson, 2004). The \textit{gsy cvp2} double mutant was additive, with similar low complexity as either of the single mutants together with non-meeting veins and vascular islands associated with the \textit{cvp2} single mutant. \textit{cvp} 2 seems to suppress \textit{gsy} in lateral roots and rosette branches but \textit{gsy} is epistatic to \textit{cvp2} in cauline branches.
DISCUSSION

It is now widely accepted that auxin has a universal role in plant development. Molecular genetic research of auxin transport and auxin signaling has yielded great insight into a wide variety of patterning processes from apical/basal polarities in young embryos to tissue patterning. We have identified a leaf vein patterning mutant called grassy (gsy) that shows a simple first leaf vein pattern with reduced numbers of secondary, tertiary and quaternary veins. Furthermore, the vascular development is delayed in gsy by about one day as suggested by temporal expression of DR5::GUS and xylem differentiation, implying that gsy acts prior to auxin response in leaf development. At each stage, gsy leaf primordia are smaller than wild type, suggesting that all aspects of leaf development are delayed. Moreover, the veins are often non-meeting and form somewhat parallel to one another and the leaves are narrow and pointed. Along with alterations to leaf phenotype, gsy shoots show increased growth of axillary buds and shortened internodes, gsy roots are shortened, show slight defects to gravitropism, have decreased formation of lateral roots, and the root hairs are elongated.

The spectrum of defects suggests that GSY has a global role in plant development likely involving auxin response or transport. The recessive nature of gsy indicates that it likely represents a loss-of-function mutation. I hypothesize that these auxin related phenotypic traits are the consequence of altered auxin transport.

gsy shows no reduction in auxin response

While many of the gsy phenotypic characteristics are consistent with defects to either auxin response or auxin transport, my analysis suggests that gsy is not defective in
auxin response. Resistance to the root growth inhibition caused by 2,4-D treatment is a standard assay for auxin resistance in auxin response mutants (Marchant et al., 1999). Roots of seedlings homozygous for \textit{axr1} are resistant to auxin inhibition of growth (Estelle and Somerville, 1987). My results show that inhibition of \textit{gsy} root elongation is not resistant but rather more sensitive to 2,4-D as compared to wild type. Lateral root formation is induced by either exogenous or endogenous shoot derived auxin and exogenous auxin results in increased formation of lateral roots in wild type (Bholero et al., 2002, Casimiro et al., 2003). Exogenous auxin restored lateral root production in \textit{gsy}. Like root elongation, lateral root development is more sensitive to 2,4-D. As compared to wild type, induction of root hair elongation in \textit{gsy} mutants by 2,4-D is greater than that in wild type. Moreover, the additive nature of the \textit{gsy axr1} double mutant suggests that \textit{GSY} and \textit{AXR1} act in independent pathway. These findings suggest that \textit{gsy} mutants are not resistant to auxin inhibition of growth. Further support for this conclusion comes from \textit{gsy rty} double mutant where \textit{rty}, an auxin over-synthesizing mutant (King et al., 2005), further simplifies the \textit{gsy} simple leaf vein pattern, reduces the overall length of primary root, increases root hair elongation and rescues lateral root production in \textit{gsy}. Together these results suggest that response to both endogenous and exogenous auxin is intact in \textit{gsy}.

Further indication that the auxin response is intact in \textit{gsy} plants is the normal level of DR5::GUS expression seen in \textit{gsy}. In \textit{DR5::GUS} construct, b-glucuronidase is controlled by synthetic auxin response element and, when assayed histochemically, provides an indication of auxin activity in the tissues (Ulmasov et al., 1997). My analysis of developing first leaf and root indicates that the \textit{gsy} mutation disrupts the temporal and
spatial arrangement of the auxin response cells but not the intensity of expression. The combination of normal intensity and change to patterning of auxin response suggests that while auxin the response is intact, transport may be defective.

**gsy is defective in auxin transport**

If the phenotypic characteristics of *gsy* including simplified leaf vein pattern, reduced root growth, defective gravitropism and reduced number of lateral roots result from defects to auxin transport, we might expect that these characteristics would be very sensitive to further chemical inhibition of auxin transport by NPA.

As my results show, the first leaf vein pattern in *gsy* is in fact more sensitive to auxin transport inhibition treatments. Similar results have been found in the *van3* mutant where the effect of an auxin transport inhibitor and of the *VAN3* mutation are additive in the formation of the venation pattern in *Arabidopsis* leaf (Koizumi et al., 2005). Moreover reduced root growth, enhanced root hair elongation, loss of gravitropism and loss of capacity to produce lateral roots in *gsy* mutants occur at relatively lower concentrations of NPA compared to wild type, suggesting that *GSY* is involved in auxin transport. I expected that *gsy* would show similar sensitivity to auxin transport mutants such as *pin1, aux1 and pin2/eir1*. Surprisingly, all auxin transport mutants tested either suppress or are suppressed by the *gsy* mutation. This suggests either that the transport activity of *GSY* acts in opposition to that of these genes, or that the defective auxin transport in *gsy* results from the over expression of these genes.
Shoot transport

A range of shoot phenotypic characters defective in gsy have previously been found to be due to defective auxin transport. gsy plants bolt and flower early with fewer rosette leaves, have increased rosette branching (axillary bud growth) pattern and produce an inflorescence with reduced internode elongation. Similar phenotypes may result from defects to auxin transport for example, plants mutant for MY2, MAX1 or MAX2 (Holweg and Nick, 2004, Stirnberg et al., 2002).

The increased rosette branching in gsy mutants suggests that axillary buds are released from apical dominance more frequently indicating that GSY negatively regulates axillary growth in Arabidopsis. Auxin is required for shoot elongation (Lomax et al., 1995) and apically derived auxin inhibits shoot branching by inhibiting the activity of axillary buds (Leyser, 2003). The increased lateral bud outgrowth and altered internode elongation of the gsy shoot is consistent with defects to basipetal auxin transport.

The branching of gsy is suppressed in either eir1 or aux1 backgrounds. One explanation is that since EIR1 and AUX act in roots, loss of either activity might lead to more auxin in the shoot due to lack of a functional sink. The increased auxin would then prevent lateral outgrowth in the double mutant. The lack of axillary meristem in pin1 is proposed to result from lack of auxin within the shoot apical meristem. The formation of axillary meristems in the gsy pin1 double mutant is most consistent with gsy showing reduced basipetal transport, which would then cause auxin to accumulate in the apex.
**Root transport**

A coordinated action of PIN proteins determines the stable pattern of auxin accumulation in the root. Since much of the auxin in the root is shoot derived, changes to shoot transport are expected to affect root transport. Two of the shoot derived auxin fluxes, polar auxin transport and phloem transport, function to mobilize shoot-derived auxin to the root apex. After reaching the root apex, auxin forms a gradient that is centred over the root apical meristem. Mutant studies have revealed that five PIN proteins (PIN1, PIN2, PIN3, PIN4 and PIN7) collectively fine tune the root apical auxin gradient (Blilou et al., 2005). The coordinated action of PIN1, PIN4 and PIN7 mediate acropetal auxin transport through the central vasculature towards the tip, PIN3 exports auxin from the meristem and finally PIN2/EIR1 transports auxin basipetally back up the sides through the epidermis (Friml, 2003, Rashotte et al., 2000). This basipetal flow of auxin has been shown to be an important component regulating cell expansion in the elongation zone. The auxin maximum at the root tip is the result of the steady state balance between the arrival and departure of auxin. A further auxin flux in the root apex, termed auxin reflux, involving the recycling of auxin from epidermal to stele tissues and back to the root apex, has been proposed (Blilou et al., 2005). This suggests that the auxin maximum in the root tip is supplied, not only by an acropetal flow of auxin from further up the root, but also by auxin being constantly recycled back into the maximum. Multiple pin mutant combinations disrupt the meristematic and cell expansion zone organization (Blilou et al., 2005) suggesting that PIN auxin efflux facilitators might be functionally important for this auxin reflux pathway.
The spectrum of gsy root phenotypic characteristics is most consistent with GSY being required for basipetal auxin transport and possibly having a role in reflux looping of the auxin. Compared to wild type, gsy plants have changes to DR5::GUS expression pattern, shorter and slightly agravitropic primary root, reduced numbers of lateral roots and increased numbers of longer root hairs.

I expected that the reduced basipetal auxin transport in gsy shoot would result in greatly reduced DR5::GUS expression in root. In fact four different patterns and levels of DR5::GUS expression are observed in the gsy root tip. Consistent with my prediction, some roots show weaker DR5::GUS expression (Figure 8g) likely due to defective auxin transport from the shoot. Other roots show a similar or higher level of expression as in wild type (Figure 8f). This can be explained either by compensatory synthesis of auxin at the root tip or by increased reflux from the basipetal stream. In many gsy roots, ectopic and asymmetric localization in the epidermal region and lateral root (Figure 8 e,f) suggests defective basipetal transport of auxin and ectopic expression at the stele (Figure 8 e,g) likely by auxin backing up in the stele, also due to defective auxin transport.

The reduced number of lateral roots in gsy mutants could either be due to reduced auxin delivery from the gsy shoot or altered auxin transport within the root itself. It is widely accepted that impairing auxin transport affects lateral roots (Reed et al., 1998) and recent studies suggest that tip-produced, basipetally transported auxin is required for the initiation of lateral root primordia, while acropetally transported auxin from the shoot is required for its subsequent growth (Casimiro et al., 2001, Bhalerao, et al., 2002). Table 7 shows a reduced number of emerged lateral roots in gsy; I observed a similar reduction in lateral root primordium as indicated by DR5::GUS maximum suggesting that lateral root
initiation is defective. A decrease in lateral root initiation is consistent with defective basipetal transport of auxin in gsy.

Auxin is a well defined positive regulator of root hair elongation. PIN2 is expressed in a non-overlapping pattern in the lateral root cap and older epidermis and cortex cells with apical (upper) polarity in the epidermis and predominantly basal polarity in the cortex (Müller et al., 1998, Friml et al., 2003). It has been shown that defects to auxin efflux carriers PIN2 or AtPGP4 (Cho et al., 2007) within epidermal cells can increase auxin in the hair and hence induce root hair elongation. The elongated root hairs seen in gsy are consistent with GSY being an auxin transporter expressed in epidermal cells.

The agravitropic root behaviour is often observed in mutants defective in auxin transport (Marchant et al., 1999, Muller et al., 1998) or signalling (Hobbie and Estelle, 1995, Nagpal et al., 2000). This bending depends on differential cell elongation resulting from differential lateral and basipetal auxin transport from the root tip back to the elongation zone (Friml, 2003, Rashotte et al., 2000). The lateral auxin gradient is transported via the lateral root cap to expanding epidermal cells through the combined activities of AUX1 and PIN2. The basipetal stream is thought to be the mechanism that delivers auxin asymmetrically from the tip after reorientation, so impairments in it might be expected to impair curvature development. Mutations in PIN2, AUX1 (Chen et al., 1998; Muller et al., 1998) or auxin transport inhibitors such as naphthylphthalamic acid (NPA) impair basipetal auxin transport and gravitropism (Muday, 2001). Consistent with the asymmetric DR5::GUS expression in gsy epidermis the primary root of gsy was
slightly deviated from the vertical axis compared to wild type (Figure 6b) further supporting the role of GSY in basipetal auxin transport.

My results overall suggest defects to basipetal transport in gsy roots. While GSY may directly affect basipetal transport, an alternative suggested by the asymmetric and sometimes overexpression of DR5::GUS is that the change in basipetal transport is the result of root tip compensation, either by synthesis or by reflux, of reduced auxin transported from the shoot.

In either case, we might now predict that GSY participates in the standard fountain model of auxin flow in roots (Swarup and Bennett, 2003) to include reflux loops that cause auxin to recirculate from epidermal and cortical cells back into the stele, where it rejoins the acropetal stream (Blilou et al., 2005). Thus GSY plays a role in forming and maintaining apical–basal auxin gradients that are essential for establishing polarity and functions in overall root auxin transport.

**Leaf transport**

The gsy mutant first leaf vascular pattern is distinct from wildtype with a reduction in number of veins and areoles and an increase in non-meeting veins. However the normal cotyledon pattern of the gsy mutant suggests that GSY acts organ specifically in vein pattern formation. The non-meeting veins leaf phenotype is consistent with altered auxin transport mutants such as fkd1, sfc40 (Steynen and Schultz, 2003, Deyholos et al., 2000). The first observable defect is the delayed leaf expansion and delayed expression of DR5::GUS at the distal tip. Auxin is essential for cell growth affecting both cell division and cellular expansion and it is likely that the delay in establishment of
**DR5::GUS** maxima at the distal tip also delays expansion. Since distal tip expression in wild type is proposed to arise through PIN directed auxin transport within the epidermis (Scarpella et al., 2006), it could be that GSY is required for PIN localization to bring about this initial transport or that GSY it works together with PIN1, like PGP (Blakeslee et al., 2007), to bring specificity and directionality to polar auxin transport. In later developmental stages, there is a continued delay in the expression of the **DR5::GUS** together with lack of expression of **DR5::GUS** in areas of distal junction. It is likely that the lack in auxin response leads to subsequent lack of vein meeting. One explanation for the lack of **DR5::GUS** expression at the junctions is that altered auxin transport results in insufficient auxin in those regions and since PIN1 is the key regulator of efflux in procambial cells (Scarpella et al., 2006), it is possible that as in the epidermis, GSY influences PIN activity. Consistent with this idea is the observation that the **pin1** phenotype is epistatic to **gsy**.

Auxin induces the differentiation of procambial cells/procambial initials from parenchymal cells (Scarpella et al., 2004). One explanation for the distal non-meeting veins in **gsy** might be that decreased transport causes reduced auxin response at distal junctions and hence a decrease in the number of procambial initials that are differentiated from parenchymal cells. The increased distance between each procambial initial in the **gsy** leaves may prevent them from connecting to one another, thus forming a discontinuous vascular network. Another explanation would be that the reduced transport results in delayed establishment of auxin response, and during this delay, mesophyll cells differentiate and interrupt the network formation of procambial cells (Scarpella et al., 2004).
The idea that $GSY$ reduces auxin transport while consistent with much of my data seems inconsistent with the mutual suppression of $gsy$ and the auxin transport mutants $eir1$ and $aux1-7$. Instead the suppression suggests either that the genes act in opposition to $GSY$ in transporting auxin or that these genes are overexpressed in absence of $GSY$. The first idea seems unlikely, since it implies that both influx and efflux proteins act in opposition to $GSY$. The second possibility suggests an alternative hypothesis that the absence of $GSY$ is compensated for by increased expression of other transporters. According to this scenario, the simplified $gsy$ phenotype would result from increased auxin transport which would increase auxin drainage from the ground meristem cells and increase the spacing between adjacent vascular strands. The increased transport could be relieved either by mutating auxin transport related genes ($EIR1, AUX1-7$). In contrast the more complex $gsy$ phenotype which is similar to $pin1$ results when compensation for the decreased auxin transport caused by $GSY$ loss does not result in increased transport.

**Conclusions.**

The overall shoot, root and leaf phenotype in $gsy$ suggests that $GSY$ is involved in auxin transport in *Arabidopsis*. In all tissues, it seems likely that the primary defect is a reduction in auxin transport. Some aspects of the root phenotype, as well as the simplified vein pattern seen in some leaves suggest that the primary reduction in auxin transport may sometimes be compensated for by increased auxin synthesis or reflux and that increased transport results from over compensation.
REFERENCES


Table 1. Primer markers developed and used in mapping gsy

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<td>1-44-b1</td>
<td>acatttggcactccacatggaagt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-44-1 F</td>
<td>aaaaagttgagacacatggaacgactgtgctgctgctg</td>
<td>CAPS (Xho I)</td>
<td>F3M18</td>
<td>453</td>
<td>56 &amp; 397</td>
</tr>
<tr>
<td>1-44-1 R</td>
<td>atgtggcagctgatgtaatttga</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-44 F</td>
<td>cagtttttctgtgaacttcaccctacccttcttgctg</td>
<td>SSLP</td>
<td>F1K23</td>
<td>399</td>
<td>361</td>
</tr>
<tr>
<td>1-44 R</td>
<td>tgcctttctgtgacactgttggaactcttgctgctg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-52 F</td>
<td>tgaaagcgaacagttgatgactttg</td>
<td>SSLP</td>
<td>F28J9</td>
<td>255</td>
<td>211</td>
</tr>
<tr>
<td>1-52 R</td>
<td>tagcggagaaaaaggattgaggagttg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* indicates lengths of final polymorphic fragment(s) of each ecotype

*a* nga 392 is a known primer marker (sequence obtained through TAIR)

*b* enzymes in bracket indicate Restriction enzymes used for digestion
## Table 2. List of candidate genes and their functions

<table>
<thead>
<tr>
<th>Genes</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G27840</td>
<td>nucleotide binding; similar to transducin family protein / WD-40 repeat family protein.</td>
</tr>
<tr>
<td>AT1G27850</td>
<td>similar to proline-rich family protein.</td>
</tr>
<tr>
<td>AT1G27860</td>
<td>ATP binding / aminoacyl-tRNA ligase; similar to ATP binding / aminoacyl-tRNA ligase.</td>
</tr>
<tr>
<td>AT1G27870</td>
<td>nucleic acid binding; similar to glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein.</td>
</tr>
<tr>
<td>AT1G27880</td>
<td>CCR4-NOT transcription complex protein, putative; similar to CCR4-NOT transcription complex protein.</td>
</tr>
<tr>
<td>AT1G27900</td>
<td>RNA helicase, putative; similar to ATP-dependent RNA helicase.</td>
</tr>
<tr>
<td>AT1G27910</td>
<td>U-box domain-containing protein; similar to armadillo/beta-catenin repeat family protein / U-box domain-containing family.</td>
</tr>
<tr>
<td>AT1G27920</td>
<td>microtubule associated protein (MAP65/ASE1) family protein.</td>
</tr>
<tr>
<td>AT1G27930</td>
<td>unknown protein.</td>
</tr>
<tr>
<td>AT1G27940</td>
<td>PGP13 (P-GLYCOPROTEIN 13); ATPase, coupled to transmembrane movement of substances; Identical to Putative multidrug resistance protein.</td>
</tr>
<tr>
<td>AT1G27950</td>
<td>lipid transfer protein-related; Identical to Uncharacterized GPI-anchored protein.</td>
</tr>
<tr>
<td>AT1G27960</td>
<td>encodes a protein whose C-terminal region is similar to ECT1 and ECT2, which bind to CIIP1.</td>
</tr>
<tr>
<td>AT1G27970</td>
<td>Encodes an ortholog of yeast NTF2, a nuclear envelop transport protein that functions as the nuclear import receptor.</td>
</tr>
<tr>
<td>AT1G27980</td>
<td>pyridoxal-dependent decarboxylase family protein; Identical to Sphingosine-1-phosphate lyase.</td>
</tr>
<tr>
<td>AT1G27990</td>
<td>unknown protein.</td>
</tr>
<tr>
<td>AT1G28000</td>
<td>similar to pentatricopeptide (PPR) repeat-containing protein.</td>
</tr>
<tr>
<td>AT1G28010</td>
<td>PGP14 (P-GLYCOPROTEIN 14); ATPase, coupled to transmembrane movement of substances; Identical to Multidrug resistance protein.</td>
</tr>
<tr>
<td>AT1G28020</td>
<td>pentatricopeptide (PPR) repeat-containing protein; similar to pentatricopeptide (PPR) repeat-containing protein.</td>
</tr>
<tr>
<td>AT1G28030</td>
<td>oxidoreductase, 2OG-Fe(II) oxygenase family protein; similar to 2-oxoglutarate-dependent dioxygenase.</td>
</tr>
<tr>
<td>AT1G28040</td>
<td>protein binding / zinc ion binding; similar to zinc finger (C3HC4-type RING finger) family protein.</td>
</tr>
<tr>
<td>AT1G28050</td>
<td>zinc finger (B-box type) family protein; Identical to Zinc finger protein CONSTANS-LIKE 15 (COL15).</td>
</tr>
<tr>
<td>AT1G28060</td>
<td>small nuclear ribonucleoprotein family protein / snRNP family protein; similar to RNA splicing factor-related.</td>
</tr>
<tr>
<td>AT1G28070</td>
<td>protein binding.</td>
</tr>
<tr>
<td>AT1G28080</td>
<td>unknown protein.</td>
</tr>
<tr>
<td>AT1G28090</td>
<td>polynucleotide adenylyltransferase family protein; similar to polynucleotide adenylyltransferase family protein.</td>
</tr>
<tr>
<td>AT1G28100</td>
<td>unknown protein.</td>
</tr>
<tr>
<td>AT1G28110</td>
<td>SCPL45; serine carboxypeptidase; Identical to Serine carboxypeptidase-like 45 precursor.</td>
</tr>
</tbody>
</table>
Table 3. Cotyledon vascular pattern characters for various genotypes at 14 DAG

<table>
<thead>
<tr>
<th></th>
<th>Secondary veins</th>
<th>Areoles</th>
<th>Non meeting veins</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (18)</td>
<td>3.5±0.71</td>
<td>3.11±0.99</td>
<td>0.38±0.50</td>
</tr>
<tr>
<td>gsy (17)</td>
<td>3.64±0.61</td>
<td>3.11±0.78</td>
<td>0.52±0.71</td>
</tr>
<tr>
<td>mp (16)</td>
<td>0.5±0.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.81±0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31±0.48&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>gsy mp (21)</td>
<td>0.95±0.66&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.33±0.58&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.61±0.66</td>
</tr>
</tbody>
</table>

Values represent means ± SEM. Number in bracket represents number of plants scored.

<sup>a</sup> The single mutant is significantly different from wild type (p<0.05).

<sup>b</sup> The double mutant is significantly different from its corresponding single mutant (p<0.05).

<sup>c</sup> The double mutant is significantly different from gsy (p<0.05).
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Secondary Veins</th>
<th>Tertiary veins</th>
<th>Quaternary veins</th>
<th>Areoles</th>
<th>Free ending secondaries (%)</th>
<th>Free ending tertiaries (%)</th>
<th>Vascular Islands</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (25)</td>
<td>8.53±0.83</td>
<td>22.53±5.47</td>
<td>4.4±1.18</td>
<td>23.6±5.13</td>
<td>0.40±0.51 (4.69)</td>
<td>7.06±3.84 (31.33)</td>
<td>0</td>
</tr>
<tr>
<td>gsy (25)</td>
<td>7.56±1.33 a</td>
<td>12.76±7.02 a</td>
<td>1.44±1.22 a</td>
<td>14.8±6.27 a</td>
<td>2.00±1.32 (26.45) a</td>
<td>4.56±2.81 (35.73) a</td>
<td>0.12±0.33</td>
</tr>
<tr>
<td>axr1-3 (25)</td>
<td>5.04±1.10 a</td>
<td>6.20±2.59 a</td>
<td>0.16±0.37 a</td>
<td>5.36±2.41 a</td>
<td>1.32±1.11 (26.19) a</td>
<td>4.24±1.76 (68.38) a</td>
<td>0.12±0.33</td>
</tr>
<tr>
<td>gsy axr1-3 (24)</td>
<td>4.87±0.94 cd</td>
<td>2.85±1.83 bcd</td>
<td>0 cd</td>
<td>1.35±1.39 bcd</td>
<td>3.78±0.97 (79.9) bcd</td>
<td>2.57±1.78 (90) bcd</td>
<td>0.07±0.26</td>
</tr>
<tr>
<td>cvp2-1 (22)</td>
<td>8.41±1.74</td>
<td>18.20±5.61 a</td>
<td>1.95±0.90 a</td>
<td>7.41±1.69 a</td>
<td>3.16±1.52 (37.57) a</td>
<td>15±5.21 (82.41) a</td>
<td>5.00±2.08 a</td>
</tr>
<tr>
<td>gsy cvp2-1 (21)</td>
<td>8.61±1.28 c</td>
<td>15.85±4.31 d</td>
<td>1.57±0.87 d</td>
<td>9.52±3.14 bcd</td>
<td>3.42±1.50 (39.72) cd</td>
<td>10.85±3.48 (66.45) bcd</td>
<td>4.42±1.98 cd</td>
</tr>
<tr>
<td>eir1-1 (22)</td>
<td>8.18±1.13</td>
<td>13.81±4.14 a</td>
<td>2.09±1.01 a</td>
<td>16.00±3.72 a</td>
<td>0.32±0.47 (3.91)</td>
<td>3.90±2.15 (28.24) b</td>
<td>0</td>
</tr>
<tr>
<td>gsy eir1-1 (18)</td>
<td>8.83±1.04 c</td>
<td>17.11±4.53 bcd</td>
<td>2.66±1.49 cd</td>
<td>20.11±4.99 bcd</td>
<td>0.83±0.71 (9.39) bcd</td>
<td>3.88±1.40 (22.67) d</td>
<td>0</td>
</tr>
<tr>
<td>aux1-7 (23)</td>
<td>7.47±0.99 a</td>
<td>10.43±2.84 a</td>
<td>1.34±0.88 a</td>
<td>13.04±3.52 a</td>
<td>0.52±0.79 (6.96)</td>
<td>4.21±2.04 (40.36) a</td>
<td>0</td>
</tr>
<tr>
<td>gsy aux1-7 (21)</td>
<td>8.42±1.07 bc</td>
<td>16.80±4.24 bcd</td>
<td>2.47±0.86 bd</td>
<td>18.90±4.32 bd</td>
<td>0.47±0.60 (5.58) c</td>
<td>4.19±1.54 (24.94) d</td>
<td>0</td>
</tr>
<tr>
<td>rty1 (14)</td>
<td>4.57±0.72 a</td>
<td>0.67±0.71 a</td>
<td>0 a</td>
<td>4.64±0.97 a</td>
<td>0.35±0.48 (7.81)</td>
<td>0.21±0.41 (33.33) a</td>
<td>0</td>
</tr>
<tr>
<td>gsy rty1 (12)</td>
<td>4.58±0.76 cd</td>
<td>0.50±0.65 cd</td>
<td>0 cd</td>
<td>4.66±1.11 cd</td>
<td>0.08±0.27 (1.82) cd</td>
<td>0.33±0.47 (66.66) cd</td>
<td>0</td>
</tr>
<tr>
<td>pin1-1 (18)</td>
<td>9.00±1.61</td>
<td>21.27±6.05</td>
<td>2.36±1.4 a</td>
<td>23.27±5.93</td>
<td>0.45±0.68 (5.05)</td>
<td>4.81±2.35 (22.64) a</td>
<td>0</td>
</tr>
<tr>
<td>gsy pin1 (20)</td>
<td>9.5±1.28 cd</td>
<td>24.5±5.04 c</td>
<td>2.90±2.7</td>
<td>29.4±6.05 bcd</td>
<td>0.30±0.45 (3.16) c</td>
<td>4.00±2.00 (16.33) d</td>
<td>0</td>
</tr>
</tbody>
</table>

Values represent means ± SD. Number in bracket represents number of plants scored.

a The single mutant is significantly different from wild type (p<0.05).
b The double mutant is significantly different from its corresponding single mutant (p<0.05).
c The double mutant is significantly different from gsy (p<0.05).
d The double mutant is significantly different from the wild type.
Table 5: Appearance of leaf vein characteristics in WT and *gsy*

<table>
<thead>
<tr>
<th></th>
<th>Midvein</th>
<th>Secondaries</th>
<th>Tertiaries</th>
<th>Areoles</th>
<th>NMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT 5DAG (28)</td>
<td>1</td>
<td>2.14±0.75</td>
<td>0</td>
<td>0.89±0.87</td>
<td>1.25±0.79</td>
</tr>
<tr>
<td><em>gsy</em> 5DAG (19)</td>
<td>0.42±0.50*</td>
<td>0.05±0.22*</td>
<td>0</td>
<td>0*</td>
<td>0.05±0.22*</td>
</tr>
<tr>
<td>WT 6DAG (18)</td>
<td>1</td>
<td>5.39±1.46</td>
<td>3.94±2.15</td>
<td>7.88±2.19</td>
<td>0.66±0.90</td>
</tr>
<tr>
<td><em>gsy</em> 6DAG (24)</td>
<td>1</td>
<td>1.95±1.12*</td>
<td>0*</td>
<td>0*</td>
<td>0.87±0.61</td>
</tr>
<tr>
<td>WT 7DAG (21)</td>
<td>1</td>
<td>6.47±1.16</td>
<td>5.23±3.68</td>
<td>9.04±3.70</td>
<td>0.28±0.46</td>
</tr>
<tr>
<td><em>gsy</em> 7DAG (20)</td>
<td>1</td>
<td>6.05±1.35</td>
<td>2.84±2.08*</td>
<td>4.84±1.50*</td>
<td>2.47±0.96*</td>
</tr>
</tbody>
</table>

Values represent mean ± S.D. Number in bracket represent number of leaves scored. Scoring was based on xylem lignification. NMS=non meeting secondaries.

* Significantly different from WT (p<0.05)
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of rosette leaves</th>
<th>Internode distance between 1st and 2nd branch (Cm)</th>
<th>Internode distance between 1st and 2nd floral node (Cm)</th>
<th>Number of leaves at bolting</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10.06±1.08 (30)</td>
<td>3.59±1.41 (30)</td>
<td>0.25±0.07 (22)</td>
<td>8.75 ± 0.78 (30)</td>
</tr>
<tr>
<td><em>gsy</em></td>
<td>5.7± 0.7a (30)</td>
<td>1.95± 0.72a (30)</td>
<td>0.95± 0.43a (22)</td>
<td>5.28 ± 0.71 a (30)</td>
</tr>
</tbody>
</table>

Values represent means ± SD. Except for leaves at bolting, all other characters were analysed at 29DAG. Numbers in bracket represent number of plants scored. 

* a significantly different from WT (p<0.05)
Table 7: Shoot and root branching in various genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of lateral roots (6 DAG)</th>
<th>Number of rosette branches</th>
<th>Number of cauline branches</th>
<th>Number of lateral branches based on expression of DR5 (8 DAG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>2.31±1.54 (20)</td>
<td>2.18±0.50 (23)</td>
<td>4.90±0.75 (23)</td>
<td>12.12±2.39 (17)</td>
</tr>
<tr>
<td>gsy</td>
<td>1.15±1.04 (16) ±</td>
<td>3.04±0.56 (22) ±</td>
<td>2.52±0.51 (22) ±</td>
<td>8.82±2.01 (19) ±</td>
</tr>
<tr>
<td>cvp2-1</td>
<td>2.66±2.19 (15) ±</td>
<td>2.13±0.63 (22) ±</td>
<td>3.50±0.67 (22) ±</td>
<td></td>
</tr>
<tr>
<td>gsy cvp2-1</td>
<td>2.06±1.24 (16) a ±</td>
<td>2.35±0.48 (20) a ±</td>
<td>2.3±0.66 (20) a ±</td>
<td></td>
</tr>
<tr>
<td>axr1-3</td>
<td>0.21±0.41 (23) a ±</td>
<td>3.31±0.71 (16) a ±</td>
<td>5.18±0.83 (16) ±</td>
<td></td>
</tr>
<tr>
<td>gsy axr1-3</td>
<td>4.19±2.64 (16) c ±</td>
<td>3.11±0.60 (17) c ±</td>
<td>5.23±0.56 (17) c ±</td>
<td></td>
</tr>
<tr>
<td>aux1-7</td>
<td>1.59±1.06 (17) ±</td>
<td>2.11±0.60 (17) ±</td>
<td>5.17±1.28 (17) ±</td>
<td></td>
</tr>
<tr>
<td>gsy aux1-7</td>
<td>2.50±1.63 (16) b c d</td>
<td>2.50±0.51 (17) c ±</td>
<td>2.41±0.61 (17) b d</td>
<td></td>
</tr>
<tr>
<td>eir1</td>
<td>0.55±0.89 (20) a ±</td>
<td>2.17±0.71 (18) ±</td>
<td>4.05±0.72 (18) ±</td>
<td></td>
</tr>
<tr>
<td>gsy eir1</td>
<td>2.57±1.35 (19) b c</td>
<td>2.35±0.58 (20) c ±</td>
<td>2.40±0.50 (20) b d</td>
<td></td>
</tr>
<tr>
<td>pin1-1</td>
<td>ND</td>
<td>1.41±0.90 (14) a ±</td>
<td>0±0 (14)</td>
<td></td>
</tr>
<tr>
<td>gsy pin1-1</td>
<td>ND</td>
<td>3.21±0.79 (18) b d</td>
<td>2.66±0.59 (18) b d</td>
<td></td>
</tr>
<tr>
<td>rty1</td>
<td>12.00±2.57 (17) a ±</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>gsy rty1</td>
<td>8.68±1.58 (16) b c d</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Values represent means ± SEM. Number in bracket represents number of plants scored

a The single mutant is significantly different from wild type (p<0.05).

b The double mutant is significantly different from its corresponding single mutant (p<0.05)

c The double mutant is significantly different from gsy (p<0.05).

d The double mutant is significantly different from WT (p<0.05).

ND: Not determined.
## Table 8: Primary root growth of seedlings exposed to 2,4-D

<table>
<thead>
<tr>
<th></th>
<th>0M</th>
<th>10^{-9}M</th>
<th>10^{-8}M</th>
<th>10^{-7}M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild Type</strong></td>
<td>26.25±5.91 (20)</td>
<td>19.5±4.70 (18)</td>
<td>5.23±5.23 (18)</td>
<td>1.84±0.68 (20)</td>
</tr>
<tr>
<td><strong>gsy</strong></td>
<td>15.45±3.21 (20)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.86±1.66 (16)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.21±0.83 (20)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.68±0.51 (20)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represent means ± SEM. Number in bracket represents number of plants scored.

<sup>a</sup> The single mutant is significantly different from wild type (p<0.05).

<sup>b</sup> Significantly different from 0M (p<0.05).
Figure 1. PCR amplification products defining molecular markers used in mapping.
A. SSLP marker (1-44) showing the banding pattern of controls (COL, LER and COL/LER F1) and gsy Ler F2 mapping samples. All F2 samples are Col except 11, 12 and 13 which are heterozygous.
B. CAPS marker (1-44-b1) showing the banding pattern of controls and gsy ler F2 population samples. Stul enzyme was used to cut the amplified product. The Ler product is cut into 2 fragments, whereas the Col product remains uncut.
Col- Columbia ecotype, Ler- Landsberg ecotype, Het- heterozygous
Figure 2A: Vascular pattern of cleared 21 DAG first leaves from wild type (a), strong gsy (b) weak gsy (c), cvp2-1 (d), gsy cvp2-1 (e), axr1-3 (f) gsy axr1-3(g), eir1-1 (h) gsy eir1-1 (i) gsy rty1(j), aux1-7 (k), gsy aux1-7 (l), pin1-1 (m) gsy pin1-1(n) rty1 (o). Scale bar: 1mm.

Figure 2B: Vascular pattern of 14 DAG cotyledons from wild type (a), gsy (b), mp (c) gsy mp (d,e). Scale bar: 1mm.
Figure 3A. Vascular pattern development in the first leaf of wild type (a-c) and gsy (d-f). First leaves of 5 DAG (a,d), 6 DAG (b,e) and 7 DAG (e,f) seedlings grown on AT media. Viewed by dark field optics on a compound microscope. Scale bar: 250μM

Figure 3B. FKD::GUS expression in 7 DAG leaves of wild type (a) and gsy (b). Viewed under dark field optics. Asterisks (*) represent regions of non meeting. Scale bar: 250μm.
Figure 4. DR5::GUS expression in developing first leaves of wild type (a-e) and gsy (f-j) 3 DAG (a,f), 4 DAG (b,g), 5 DAG (c,h), 6 DAG (d,i) and 7 DAG (e,j) first leaves from seedlings grown on AT media. Leaves stained for 6 hours. Viewed with differential interference contrast optics. Asterisks (*) represent regions of non meeting. Scale bar: 250μM.
Figure 5. Whole plant phenotype of various genotypes at 35 DAG. wild type (a), gsy (b), eir1-1 (c), gsy eir1-1 (d), pin1-1 (e) gsy pin1-1 (f), aux1-7 (g) gsy aux1-7 (h) cvp2-1 (i), gsy cvp2-1 (j), axr1-3 (k), gsy axr1-3 (l). Photos taken by Nikon Coolpix 990. Scale: 2.5 cm
Figure 6. Root gravitropic assay for wild type (a) and gsy (b) on 6DAG seedlings grown vertically on AT plates. Note the root bending in some gsy (arrows in b). Viewed by translumination on a dissecting scope. Scale bar: 1 cm.
Figure 7 A. 6 DAG root hairs of gsy (a) and wild type (b). Seedlings grown vertically on AT media.

Figure 7B. Root hairs of 8 DAG wild type (a-e) and gsy (f-k) primary roots. Seedlings were grown in AT media supplemented with 0 M (a,f), 5 uM (b,g), 10 uM (c,h), 30 uM (d,i), 100 uM NPA (f,j,k). Viewed by translumination on dissecting microscope.
Scale bar: 500μm
Figure 8. DR5::GUS expression in 5 DAG roots of WT (a-d) and gsy (e-h) grown on unsupplemented AT media (a, b, e, f, g) and AT media supplemented with $10^{-7}$ M 2,4-D (c, d, h). Roots were stained for 4 hours. Viewed by phase contrast optics. Scale: 100μm
Figure 9. DR5::GUS expression of 6 DAG wild type (a-f) and gsy (g-l) roots. Seedlings grown on various concentrations of NPA, 0 M (a,g), 1 μM (b,h), 5 μM (c,i), 10 μM (d,j), 30 μM (e,k) and 100 μM NPA (f,l) and roots were dissected, Viewed by dark field optics on a compound microscope. Scale bar: 100μm
Figure 10: Vascular pattern and DR5::GUS expression of 10 DAG wild type (a-e) and gsy (f-j). Seedlings grown on various concentrations of NPA. 0 M (a,f), 5 μM (b,g), 10 μM (c,h), 30 μM (d,i) and 100 μM NPA (e,j), Viewed with differential interference contrast optics on a compound microscope. Scale bar: 500μm
Figure 11. Roots of 9 DAG seedlings of wild type (a-d) and gsy (e-h) treated with 0M 2,4-D (a,e) $10^{-9}$ M 2,4-D, $10^{-8}$ M 2,4-D and $10^{-7}$ M 2,4-D. Seedlings were grown in unsupplemented AT media for five days and transferred to AT media supplemented with 2,4-D. Viewed by translumination on dissecting microscope. Scale bar: 500μm
Figure 12. Roots of 8 DAG (a-l) and 15 DAG (m,n) seedlings showing root hairs: WT (a), gsy (b), cvp2 (c), gsy cvp2 (d), axr1 (e), gsy axr1 (f), pin1 (g) gsy pin1 (h), aux1-7 (i) gsy aux1-7 (j), eir1 (k), gsy eir1 (l), rty1 (m) gsy rty1 (n). Viewed by translumination on dissecting microscope. Scale Bar: 0.5mm